Epigenética. Reversão da hipermetilação e reativação de vários genes supressores de tumor no câncer com o emprego da genisteína

Reversal of hypermethylation and reactivation of p16INK4a, RARβ, and MGMT genes by genistein and other isoflavones from soy.
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Experimental Design: Enzyme assay was used to determine the inhibitory effect of genistein on DNA methyltransferase activity in nuclear extracts and purified recombinant enzyme. Methylation-specific PCR and quantitative real-time PCR were employed to examine the DNA methylation and gene expression status of retinoic acid receptor beta (RARβ), p16INK4a, and O6-methylguanine methyltransferase (MGMT) in KYSE 510 esophageal squamous cell carcinoma cells treated with genistein alone or in combination with trichostatin, sulforaphane, or 2′-deoxy-5-aza-cytidine (5-aza-dCyd).

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Conclusions: These results indicate that genistein and related soy isoflavones reactivate methylation-silenced genes, partially through a direct inhibition of DNA methyltransferase, which may contribute to the chemopreventive activity of dietary isoflavones.

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detoxification enzymes (1, 2). Unlike many other genomic alterations that occur during carcinogenesis, DNA methylation is potentially reversible via the use of preventive or therapeutic agents (3, 4). S-Aza-cytidine and 2′-deoxy-5-aza-cytidine (5-aza-dCyd), nucleoside analogue inhibitors of DNA methyltransferases, have been widely used in attempts to reverse abnormal DNA hypermethylation in cancer cells and restore “silenced” gene expression. However, clinical utility of these nucleoside analogue DNA methyltransferase inhibitors has been somewhat limited by myelosuppression and other side effects (5). We have previously shown the reactivation of methylationsilenced genes with the use of the DNA methyltransferase inhibitor, 5-aza-dCyd, in order to determine the role of methylation status in prostate cancer cells (6). Now we report the in vivo effect of genistein, a naturally occurring soy isoflavone, on the expression of methylated genes in prostate cancer cells. Genistein is a potential nutritional factor that may influence prostate cancer development (7). The effects of genistein on various cancer cells have been extensively studied. It has been reported that genistein can inhibit cancer cell growth (21–23), induce apoptotic cell death accompanied by cell cycle arrest at G2-M phase, and inhibit angiogenesis (17, 24–28).

Previously, we tested the ability of soy isoflavones to inhibit DNA methylation. The precise molecular mechanisms responsible for these activities, however, are not clearly known. One potential mechanism that has recently received considerable attention is that genistein may be involved in regulation of gene activity by modulating epigenetic events such as DNA methylation and/or histone acetylation directly or through an estrogen receptor dependent process (29–31). This hypothesis is supported by a report indicating that dietary genistein causes epigenetic changes in mouse prostate (32). Genistein can also up-regulate mRNA expression of the BRCA1 gene during mammary tumorigenesis, which is frequently inactivated by epigenetic events in breast cancer (33).

In this preclinical study, we examined the effect of genistein and its combination with other agents on the reactivation of methylation-silenced RARβ, p16, and MGMT genes, as well as its effect on the activity of DNA methyltransferase and histone deacetylase (HDAC) in the esophageal squamous cell carcinoma cell line KYSE 510. These genes were selected for study because they have been shown to be progressively inactivated by DNA hypermethylation in human esophageal squamous carcinogenesis and to be reactivated in KYSE 510 cells by (−)-epigallocatechin-3-gallate (EGCG) and 5-aza-dCyd (33–35). Our results show that genistein inhibits DNA methyltransferase and reverses the methylation status, with accompanying reexpression of RARβ, p16, and MGMT genes. These activities may contribute to the chemopreventive activity of genistein.

**Materials and Methods**

**Cell mass and cell culture**

The human esophageal squamous cell carcinoma cell lines KYSE 510 and KYSE 150 were a gift from Dr. Yutaka Shimada (Kyoto University, Kyoto, Japan). The cells were maintained in 5% CO2 atmosphere at 37°C in RPMI 1640/Ham’s F-12 mixed (1:1) medium containing 5% fetal bovine serum. To determine the dose-dependent changes, KYSE 510 cells were treated with 2, 5, 10, or 20 µM of genistein (Sigma, St. Louis, MO) or 0.7 µM of 5-aza-dCyd (Sigma) administrated in fresh culture medium every other day, when the medium was changed, for 6 days. For the time-course study, cells were treated with 5 µM of genistein in fresh culture medium on days 0, 2, 4, and 5. Prostate cancer cell lines LNCaP and PC3 were obtained from American Type Culture Collection (Manassas, VA) and were grown in RPMI 1640 containing 10% fetal bovine serum. KYSE 150, LNCaP, and PC3 cells were treated with 10 or 20 µM of genistein for 6 days as described above. For the combination study, KYSE 510 cells were treated with either 5 µM of genistein or 2 µM of 5-aza-dCyd for 5 days and then cultured for 1 additional day in fresh medium with 0.5 µM of trichostatin A or 15 µM of sulforaphane; or treated with 5 µM of genistein or 2 µM of 5-aza-dCyd alone or together for 5 days and 1 additional day in fresh medium.

**Bisulfite modification and methylation-specific PCR**

DNA was extracted from the cells using the DNeasy tissue kit (Qiagen, Valencia, CA) following the procedure of the manufacturer and used as the source of methylated DNA. Primers specific for the methylated and unmethylated DNA were the same as we previously reported (6). To verify the specificity of the PCR, we used normal human placental DNA (Sigma) as a negative control and CpGenome universal methylated DNA (Chemicon, Temecula, CA) as a positive control for methylation-specific PCR.

Methylation-specific PCR was carried out using a nested two-stage PCR approach (37) with similar PCR conditions as previously described (6). In brief, stage 1 PCR was done on bisulfite-modified DNA to amplify the CpG-rich promoter regions of the p16, RARβ, or MGMT gene with the primers that recognize the bisulfite-modified template, but do not discriminate between methylated and unmethylated alleles. DNA fragments from the first PCR were used for methylation-specific PCR with primers specific for methylated and unmethylated DNA. Amplification was carried out using a 9700 Perkin-Elmer thermal cycler (Applied Biosystems, Foster City, CA).

**Reverse transcription-PCR**

Total RNA was isolated from cells using TRI reagent (Sigma). Reverse transcription of RNA was done using the Advantage RT-for-PCR kit (Clontech, Palo Alto, CA). The primers and conditions used in the PCR for the genes analyzed were previously summarized (6). The PCR was carried out in an approximate linear range (for example, 25 to 28 cycles for p16, 30 to 35 cycles for RARβ, and 30 to 35 cycles for MGMT). The PCR products were separated on 2% agarose gel containing ethidium bromide and then photographed. Negative controls for PCR were run under the same conditions without RNA or reverse transcriptase. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA was used as an endogenous control.

**DNA methyltransferase assays**

DNA methyltransferase activities were assayed as previously described by Herman et al. (36). In brief, stage I PCR was done on bisulfite-modified DNA to amplify the CpG-rich promoter regions of the p16, RARβ, or MGMT gene with the primers that recognize the bisulfite-modified template, but do not discriminate between methylated and unmethylated alleles. DNA fragments from the first PCR were used for methylation-specific PCR with primers specific for methylated and unmethylated DNA. Amplification was carried out using a 9700 Perkin-Elmer thermal cycler (Applied Biosystems, Foster City, CA).

**DNA methyltransferase assays**

DNA methyltransferase assays were prepared with a nuclear extraction kit (Pierce, Rockford, IL). The DNA methyltransferase assay was modified in our lab according to the published methods (40, 41). In brief, the nuclear extracts (4.5 µg of protein) were incubated for 1.5 hours at 37°C with 20 µM of genistein or 8.7 µM of 5-aza-dCyd (Sigma) administered in fresh culture medium every other day, when the medium was changed. The nuclear extracts (4.5 µg of protein) were incubated for 1.5 hours at 37°C with 20 µM of genistein or 8.7 µM of 5-aza-dCyd (Sigma) administered in fresh culture medium every other day, when the medium was changed. The nuclear extracts (4.5 µg of protein) were incubated for 1.5 hours at 37°C with 20 µM of genistein or 8.7 µM of 5-aza-dCyd (Sigma) administered in fresh culture medium every other day, when the medium was changed. The nuclear extracts (4.5 µg of protein) were incubated for 1.5 hours at 37°C with 20 µM of genistein or 8.7 µM of 5-aza-dCyd (Sigma) administered in fresh culture medium every other day, when the medium was changed.
was dissolved in DMSO, and all incubations were adjusted to contain an equivalent concentration of DMSO (10%), which had no significant effect on DNA methyltransferase activity. Reactions were initiated by the addition of nuclear extracts and stopped by mixing with 200 µL of a solution containing 1% SDS, 3% 4-aminosalicylate, 5% butanol, 2.0 mM EDTA, 125 mM/L NaCl, 0.25 mg/mL carrier salmon testes DNA (Life Technologies, Inc., Gaithersburg, MD), and 1 mg/mL protease K. DNA was extracted using the phenol/chloroform method, purified with ethanol precipitation, and then washed thrice with 70% ethanol. The radioactivity in the pellets was counted in a scintillation counter. Each assay was done in duplicate. Background levels were determined in incubations without the template poly(dI-dC)·poly(dI-dC). For kinetics, the results were analyzed using GraphPad Prism 4 (GraphPad Software, San Diego, CA). Assays with purified recombinant DNA methyltransferase 1. Recombinant murine DNA methyltransferase 1 (DNMT1) with an NH2-terminal hexahistidine tag added for purification was expressed in Spodoptera frugiperda 9 cells and purified as previously described (42). Standard reaction mixtures (25 µL) contained 60 mM/L recombinant DNMT1, 15 µM/L S-adenosyl-L-[methyl-3H]methionine (specific activity, 275 GBq/mmol), and 10 ng poly(dI-dC)·poly(dI-dC) (average chain length: 3,000) in M2 buffer [100 mM/L Tris (pH 8), 10 mM/L EDTA, 10 mM/L DTT, 200 µg/mL bovine serum albumin] and incubated for 1 hour at 37°C. Reactions were terminated by the addition of NaOH to a final concentration of 0.3 mol/L. Salmon sperm DNA (100 µg/mL) was added as a carrier, and the solution brought to 860 mM/L with perchloric acid to precipitate DNA. Precipitated DNA was collected and washed on glass fiber filters before analysis for 3H-methyl by liquid scintillation counting with aqueous fluor. To measure the effect of genistein on the rate of substrate methylation, sets of duplicate reactions were initiated by adding poly(dI-dC)·poly(dI-dC) in the presence of increasing concentrations of EGCG or genistein dissolved in DMSO as described above. Cell growth inhibition and other studies KYSE 510 cells were treated with 1, 2, 5, 10, and 20 µM/L genistein, biochenin A, or daidzein added in fresh culture medium every other day or on the other day for 6 days or with 5 µM/L genistein for 1, 2, 4, or 6 days. Living cells were counted after staining with 0.04% trypan blue. After treatment for 2 or 6 days, the cells were photographed under an inverted microscope for morphologic analysis. In other experiments, KYSE 510 cells were treated with 2, 5, 10, and 20 µM/L genistein for 3 days; cell proliferation was determined with the BrdUrd ELISA method according to the instruction manual (Roche Diagnostics Co., Indianapolis, IN) and apoptosis was determined with fluorescence 4′,6-diamidino-2-phenylindole staining or caspase-3 immunocytochemistry staining. For colony formation efficiency assay, KYSE 510 cells were treated with 2, 5, 10, or 20 µM/L genistein for 2 days and then cultured for 10 additional days in fresh medium, and then colony numbers were analyzed. The effect of genistein on HDAC activity was carried out according to the instruction manual of the HDAC Assay Kit (Fluorometric Detection, Upstate Biotechnology, Lake Placid, NY). In brief, the reaction mixture contained nuclear extract from KYSE 510 cells (4.0 µg protein), substrate (CH3-CO-NH-CH2-(CH2)3-Lys-X2-, 48 µmol/L), and genistein (5, 10, 20, 50, or 100 µmol/L) in 40 µL total volume. Trichostatin (1 µmol/L) was used as a positive control. After incubation for 30 minutes at 25°C, activator solution was added to release a fluorophore from the deacetylated substrate. The product was determined by a fluorescence plate reader (excitation, 360 nm; emission, 465 nm). Each assay was done in triplicate. Statistical analyses Statistical significance between treatment and control groups was evaluated using the Student’s t test. One-way ANOVA and Tukey’s honest significant difference test were used for comparing the effects of different treatments. Pearson’s correlation coefficient (r) with P value was also determined to examine the association between concentration and efficacy. All statistical analyses were done using GraphPad Instat. Previous SectionNext Section Results Reversal of hypermethylation and reactivation of RARβ, p16, and MGMT by genistein KYSE 510 cells, with hypermethylated RARβ, p16, and MGMT genes, showed only the methylation-specific bands of these genes in methylation-specific PCR, with the loss of the respective mRNA expression, as we previously reported (6, 34). After treating the cells with 2, 5, 10, or 20 µM/L genistein for 6 days, the unmethylation-specific bands of these three genes were detectable at 2 µM/L, with higher intensity at 5 µM/L and even higher intensity at 10 and 20 µM/L (Fig. 1A). The expression of mRNA from all three genes increased approximately proportional to the appearance of unmethylated DNA, whereas PCR products from methylated DNA decreased as genistein concentration increased. The reversal of hypermethylation and reexpression of these three genes by genistein were similar to that produced by the classic DNA methyltransferase inhibitor 5-aza-dCyd. On treating the cells with 5 µM/L genistein, PCR products from unmethylated RARβ and MGMT genes began to appear after 2 days, whereas that from methylated p16 was only observed after 6 days. With 2, 5, 10, or 20 µM/L of genistein for 6 days, the expression of mRNA from all three genes was increased by treatment with 5 µM/L of genistein and increased further with higher concentrations of genistein (10 or 20 µM/L). However, genistein was not as effective as 5-aza-dCyd (8.7 µM/L). This result was generally consistent with our previous findings (35) of complete demethylation of RARβ gene in KYSE 150, LNCaP, and PC3 cells. Cells were treated for 1, 2, 4, and 6 days with 5 µM/L of genistein or 8.7 µM/L of 5-aza-dCyd (DAC) for 6 days. Right, the cells were treated for 1, 2, 4, and 6 days with 5 µM/L of genistein. B, alterations of methylation status and mRNA expression levels. Left, KYSE 510 cells were treated with 2, 5, 10, or 20 µM/L of genistein or 8.7 µM/L of 5-aza-dCyd (DAC) for 6 days. Right, the cells were treated for 1, 2, 4, and 6 days with 5 µM/L of genistein. B, alterations of methylation status and mRNA expression levels of RARβ gene in KYSE 150, LNCaP, and PC3 cells. Cells were treated with 10 or 20 µM/L of genistein for 6 days. To determine whether the reactivation of methylation-silenced genes by genistein is a general phenomenon and occurs in other cell lines, we evaluated the effects of genistein treatment on the methylation status and mRNA expression levels of RARβ in three other human cancer cell lines (Fig. 1B). The appearance of an unmethylation-specific band and mRNA band of RARβ in KYSE 150 was observed after treating the cells with 10 or 20 µM/L genistein for 6 days. In the LNCaP and PC3 cells, unmethylation-specific band and mRNA band of RARβ only appeared after treating the cells with 20 µM/L genistein for 6 days. Methylation-specific bands in all three cell lines were slightly decreased by treatment with 20 µM/L of genistein. The results show that the reactivation of methylation-silenced genes by genistein does occur in different cell lines as a general phenomenon, although the effective concentrations are somewhat different in the different cell lines. To confirm the effect of genistein on the reactivation of these genes, real-time quantitative PCR with TaqMan MGB detector was employed to determine the mRNA expression levels of RARβ, p16, and MGMT genes in the KYSE 510 cells after treatment with different concentrations of genistein. The results are summarized in Fig. 2. The results show that the relative amount of mRNA expression from these three genes was increased by treatment with 5 µM/L of genistein and increased further with higher concentrations of genistein (10 or 20 µM/L). However, genistein was not as effective as 5-aza-dCyd (8.7 µM/L). This result was generally consistent with those from general reverse transcription-PCR (RT-PCR) shown in Fig. 1A. View larger version: This page in a new window Download as Powerpoint Slide Fig. 1. Alterations of methylation status and mRNA expression levels of RARβ, p16, and MGMT genes after treatment of genistein. Methylation status and mRNA expression levels determined with methylation-specific PCR and RT-PCR, respectively. KYSE 510 cells were treated with 2, 5, 10, or 20 µM/L genistein or 8.7 µM/L of 5-aza-dCyd (DAC) for 6 days. Right, the cells were treated for 1, 2, 4, and 6 days with 5 µM/L of genistein. B, alterations of methylation status and mRNA expression levels. Left, KYSE 510 cells were treated with 2, 5, 10, or 20 µM/L of genistein or 8.7 µM/L of 5-aza-dCyd (DAC) for 6 days. Right, the cells were treated for 1, 2, 4, and 6 days with 5 µM/L of genistein. B, alterations of methylation status and mRNA expression levels of RARβ gene in KYSE 150, LNCaP, and PC3 cells. Cells were treated with 10 or 20 µM/L of genistein for 6 days. 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using real-time PCR with TaqMan MGB system. The results were analyzed using a comparative Ct method. β-Actin was used as an endogenous control. Each sample was run in triplicate. A, amplification plots of RARβ and β-actin are shown on right and left side, respectively. Y axis, ∆Rn = Rn – baseline (Rn, the normalized reporter). X axis, amplification cycle number. B, relative quantification for mRNA expression levels of p16, RARβ, or MGMT. Columns, mean (n = 3); bars, SD. Genistein significantly induced reexpression of p16, RARβ, and MGMT in a concentration-dependent pattern (r = 0.82-0.87, P < 0.0001).

To compare the effects of different isoflavones from soy on the reactivation of RARβ gene, KYSE 510 cells were treated with 5, 10, or 20 µmol/L of genistein, biochanin A, or daidzein for 6 days and mRNA levels of RARβ were determined by RT-PCR (Fig. 3). All three compounds caused the reexpression of RARβ mRNA, but biochanin A and daidzein were significantly less effective than genistein (P < 0.05).

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Fig. 3.

Induction of RARβ mRNA expression by different isoflavonoids. KYSE 510 cells were treated with different concentrations of genistein, biochanin A, or daidzein for 6 days, and then the RARβ mRNA levels were determined with RT-PCR. G3PDH was used as an internal control. Representative of three independent experiments. The band intensity was determined with densitometry; columns, mean (n = 3); bars, SD. Different letters indicate a significant difference (P <0.05) based on one-way ANOVA and Tukey's honest significant difference test.

Inhibition of DNA methyltransferase and histone deacetylase activities

Genistein exhibited a dose-dependent inhibition of DNA methyltransferase activity with nuclear extracts from KYSE 510 cells as the enzyme source and poly(dI-dC)•poly(dI-dC) as the substrate. The IC50 of genistein was ~67 µmol/L. The structural analogues of genistein, biochanin A, and daidzein had weaker inhibitory activities than genistein (Fig. 4A). Genistein also showed a dose-dependent inhibition effect on recombinant DNMT1 activity with an IC50 of 30 µmol/L (Fig. 4B). In kinetic studies with varying concentrations of poly(dI-dC)•poly(dI-dC), genistein decreased Vmax and increased Km, showing a mixed inhibition with a Ki of 189.3 µmol/L for competitive and a Ki of 80.2 µmol/L for noncompetitive actions (Fig. 4C). With different concentrations of S-adenosyl-L-[methyl-3H]methionine, genistein decreased Vmax without a significant change of Km, suggesting a noncompetitive inhibition with a Ki of 140.5 µmol/L (Fig. 4D). The same conclusion was reached when this experiment was repeated thrice.

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Fig. 4.

Fig. 5.

Inhibition of HDAC activity by genistein. A, dose-dependent inhibition of DNA methyltransferase activity of nuclear extracts from KYSE 510 cells by genistein, biochanin A, and daidzein. The reaction mixture contained nuclear extracts (4.5 µg protein), poly(dI-dC)•poly(dI-dC) (20 µmol/L), and S-adenosyl-L-[methyl-3H]methionine (10 µmol/L, 2.0 µCi) in 40 µL incubation mixture containing 10% glycerol and 2 mmol/L 2-mercaptoethanol. The incubation time was 1 hour. B, dose-dependent inhibition of recombinant DNMT1 activity. Standard reaction mixtures (25 µL) contained 60 µmol/L recombinant DNMT1, 15 µmol/L S-adenosyl-L-[methyl-3H]methionine (specific activity, 275 GBq/mmol), and 10 ng poly(dI-dC)•poly(dI-dC) (average chain length: 3,000) in M2 buffer [100 mmol/L Tris (pH 8), 10 mmol/L EDTA, 10 mmol/L DTT, 200 µg/mL bovine serum albumin] and were incubated for 1 hour at 37°C. C, kinetic study with varying concentrations of poly(dI-dC)•poly(dI-dC). The reaction mixtures contained 20 µmol/L of S-adenosyl-L-[methyl-3H]methionine and different concentrations of poly(dI-dC)•poly(dI-dC). Kin, competitive inhibition constant; D, kinetic study with varying concentrations of S-adenosyl-L-[methyl-3H]methionine. The reaction mixtures contained 120 µmol/L poly(dI-dC)•poly(dI-dC) and different concentrations of S-adenosyl-L-[methyl-3H]methionine. Points, mean of three sets of the same experiments; bars, SD, *, P < 0.05; **, P < 0.01, statistically significant difference from control according to Student's t test. Genistein, biochanin A, and daidzein significantly inhibited DNA methyltransferase activity of nuclear extracts and recombinant DNA methyltransferase in a concentration-dependent pattern (r = −0.95 to −0.98, P < 0.0001).

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Fig. 5.

Fig. 6.

Inhibition of HDAC activity by genistein. The reaction mixture contained nuclear extract from KYSE 510 cells (40.0 µg protein), substrate (48 µmol/L), and genistein (5, 10, 20, 50, or 100 µmol/L) in 40 µL total volume. Trichostatin (1 µmol/L) was used as a positive control. Incubation time is 30 minutes at 25°C. The product was determined by a fluorescence plate reader (excitation, 360 nm; emission, 465 nm). Columns, mean (n = 3); bars, SD, *, P < 0.05; **, P < 0.01, statistically significant difference from control according to Student's t test. Genistein inhibited HDAC activity in a concentration-dependent pattern (r = −0.89, P < 0.0001).

Enhanced reactivation of RARβ, p16, and MGMT and growth inhibition by combination of genistein with trichostatin, sulforaphane, or 2′-deoxy-5-aza-cytidine

Treatment of KYSE 510 cells with trichostatin (0.5 µmol/L) alone for 1 day or with 5-aza-dCyd (2 µmol/L) alone for 5 days reactivated the RARβ gene, but had little or no effect on p16 and MGMT (Fig. 6A). However, treatment of the cells with 5 µmol/L genistein for 5 days significantly increased with 0.5 µmol/L trichostatin for 1 additional day significantly enhanced the reactivation of these three genes. In combination with 2 µmol/L 5-aza-dCyd, genistein also significantly enhanced the reexpression of RARβ, p16, and MGMT, but not MGMT. The reaction caused by the combination of genistein with trichostatin or 5-aza-dCyd was higher than the sum of the effect of individual agents, suggesting a synergistic action. The combination of 2 µmol/L 5-aza-dCyd and 0.5 µmol/L trichostatin seemed to produce an additive effect on RARβ and a synergistic effect on p16 and MGMT. Under these experimental conditions, trichostatin (0.5 µmol/L) was more effective than genistein (5 µmol/L) in inhibiting cell growth, and the combination of these two agents caused a more pronounced inhibition (48%, P < 0.05). Similarly, 15 µmol/L sulforaphane alone can reactivate the RARβ gene, but cannot induce reexpression of p16 and MGMT genes; however, treatment of the cells with 5 µmol/L genistein for 5 days and then with 15 µmol/L sulforaphane for 1 additional day significantly enhanced the reactivation of these three genes, especially p16 and MGMT, whereas the combination of genistein and sulforaphane significantly enhanced the inhibitory effect on cell growth (P < 0.05) compared with genistein or sulforaphane alone (Fig. 6B).

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Fig. 6.

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Effects of combination of genistein and trichostatin, sulforaphane, or 5-aza-dCyd on the reactivation of p16, RARβ, and MGMT genes and cell growth. A, KYSE 510 cells were treated with or without either 5 µmol/L genistein or 2 µmol/L 5-aza-dCyd alone or together for 5 days and cultured for 1 additional day in fresh medium with or without 0.5 µmol/L trichostatin (TSA). B, KYSE 510 cells were treated with or without 5 µmol/L genistein for 5 days, and cultured for 1 additional day in fresh medium with or without 15 µmol/L sulforaphane (SFN). The mRNA expression levels of p16, RARB, and MGMT genes were determined by RT-PCR and the band intensity was quantified using densitometry and normalized to each control (mean ± SD, n = 2). Representative of two independent experiments. Cell growth was analyzed using trypan blue exclusion assay. Different letters indicate a significant difference (P < 0.05) based on one-way ANOVA and Tukey's honest significant difference test (mean ± SD, n = 3).

Effects on inhibition of cell growth and other factors

After treatment with genistein, biochanin A, or daidzein for 6 days, a dose-dependent inhibition of cell growth was observed (Fig. 7A). At 5 µmol/L, it significantly inhibited cell growth by ∼30%, and at 20 µmol/L, by more than 90%. The inhibitory effects of biochanin A and daidzein were weaker, with biochanin A the weakest. In a time-dependent study with 5 µmol/L genistein, slight growth inhibition was observed after 4 days and significant inhibition was observed after 6 days (Fig. 7A). Signs of toxicity and growth inhibition were observed in cells treated for 2 or 6 days with 10 or 20 µmol/L genistein; the greatest effect was observed in cells treated with 20 µmol/L genistein for 6 days (Fig. 7B). In cells treated with different concentrations of genistein for 5 days, cell proliferation was significantly inhibited after treatment with 10 or 20 µmol/L genistein (Fig. 7C), but induction of apoptosis was not observed even after treatment with 20 µmol/L genistein (data not shown). In colony formation assays of KYSE 510 cells, treatment with genistein, especially at 10 or 20 µmol/L, for 2 days, significantly decreased the number of colonies formed (Fig. 7D).

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Fig. 7. Effects of genistein on cell growth, morphologic change, cell proliferation, and colony formation efficiency assay. A, KYSE 510 cells were treated with different concentrations of genistein, biochanin A, or daidzein for 6 days, with or without 5 µmol/L genistein for different time periods, and then their effects on cell growth were determined by trypan blue exclusion assay. Genistein, biochanin A, and daidzein significantly inhibited cell growth with regard to concentration (r = −0.94 to −0.99, P < 0.0001) and time (r = −0.65, P = 0.0017). B, after treatment for 2 days (a-c) or 6 days (d-f), the morphology of the cells treated with 0 µmol/L (a and d), 10 µmol/L (b and e), or 20 µmol/L (c and f) genistein was observed. Magnification 1×. C, the proliferation assay showed significant inhibition of cell proliferation by genistein for 3 days, and then cell proliferation was determined with the BrdUrd ELISA method. Genistein significantly inhibited cell proliferation in a concentration-dependent pattern (r = −0.85, P < 0.0001). D, KYSE 510 cells were treated with different concentrations of genistein for 2 days and cultured for 10 additional days in fresh medium, and then colony numbers were analyzed. Genistein significantly inhibited colony formation efficiency in a concentration-dependent pattern (r = −0.96, P < 0.0001). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Student's t test on RT-PCR results with total RNA of KYSE 510 cells treated with 1, 2, 5, 10, and 20 µmol/L genistein for 6 days indicated that genistein treatment did not affect the mRNA expression levels of DNMT1, DNMT3a, DNMT3b, and methyl-CpG binding domain 2. The protein level of 5-methylcytosine DNA glycosylase, an enzyme that may be involved in the removal of methylated DNA (43), also did not change after treatment with 10 or 20 µmol/L of genistein for 24 hours (data not shown).

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Discussion

The present study clearly shows that genistein reverses DNA hypermethylation and reactivates the methylation-silenced genes RARβ, p16, and MGMT. This activity is similar to our previous observations with EGCG. Genistein is also an inhibitor of DNA methyltransferase activity in nuclear extracts from KYSE 510 cells, with 14% inhibition at 20 µmol/L (IC50, ∼67 µmol/L), and this activity is weaker than that of EGCG (IC50, ∼20 µmol/L). Kinetic studies indicate that genistein inhibits DNA methyltransferase activity in a substrate- and methyl donor-dependent manner, which differs from our previous results with EGCG as a competitive inhibitor of DNA methyltransferase. The inhibition activity of genistein was also observed using recombinant DNMT1 as the enzyme source, showing an IC50 of 30 µmol/L. In comparison with biochanin A and daidzein are weaker inhibitors of DNA methyltransferase, and they are also less effective in the reactivation of RAR gene, suggesting a correlation between the inhibition of DNA methyltransferase activity and reactivation of methylation-silenced genes by these dietary isoflavonoids. However, the fact that the effective concentrations of genistein in the inhibition of DNA methyltransferase are higher than those for the reactivation of methylation-silenced genes suggests that other factors are involved in the action of genistein. Our results, however, suggest genistein did not exert its effect by modulating the expression levels of DNMT1, 3a, and 3b, methyl-CpG binding domain 2, and 5-methylcytosine DNA glycosylase.

For the DNA hypermethylation-related silenced genes RARβ, p16, and MGMT, KYSE 510 cells were treated with different concentrations of genistein for 3 days, and then cell proliferation was determined with the BrdUrd ELISA method. Genistein significantly inhibited cell proliferation in a concentration-dependent pattern (r = −0.85, P < 0.0001). D, KYSE 510 cells were treated with different concentrations of genistein for 2 days and cultured for 10 additional days in fresh medium, and then colony numbers were analyzed. Genistein significantly inhibited colony formation efficiency in a concentration-dependent pattern (r = −0.96, P < 0.0001). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Student's t test on RT-PCR results with total RNA of KYSE 510 cells treated with 1, 2, 5, 10, and 20 µmol/L genistein for 6 days indicated that genistein treatment did not affect the mRNA expression levels of DNMT1, DNMT3a, DNMT3b, and methyl-CpG binding domain 2. The protein level of 5-methylcytosine DNA glycosylase, an enzyme that may be involved in the removal of methylated DNA (43), also did not change after treatment with 10 or 20 µmol/L of genistein for 24 hours (data not shown).

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References


